

COMPARISON OF ETHANOL YIELD BETWEEN SEPARATE AND SIMULTANEOUS HYDROLYSIS AND ETHANOL FERMENTATION OF FORMIC- FRACTIONATED SUGARCANE BAGASSE

Ngo Duy Sa¹, To Kim Anh², Pham Tuan Anh^{2,*}

¹*Department of Food Technology, Hatay Community College, Thuy Xuan Tien, Chuong My, Hanoi, Vietnam*

²*School of Biotechnology - Food Technology, Hanoi University of Science and Technology, No 1 Dai Co Viet Road, Hanoi, Vietnam*

*Email: anh.phamtuan2@hust.edu.vn

Received: 1 April 2016; Accepted for publication: 15 June 2016

ABSTRACT

The fractionation of sugarcane bagasse using formic acid allowed removing lignin and hemicellulose, obtaining a material containing up to 90 % cellulose. The material can be easily hydrolyzed into glucose to serve as materials to produce high value added products such as biofuel, chemicals, pharmaceuticals, food additives, and the likes. The hydrolysate of fractionated bagasse was easily fermented with a (ethanol) fermentation yield attained 91.08 ± 2.02 %, showing no significant inhibition to the yeast in the hydrolysate. In this study, a process of simultaneous hydrolysis and fermentation (SSF) was performed to convert fractionated sugarcane bagasse at 20 % consistency to ethanol. The process with 6h pre-hydrolysis at 50 °C then SSF at 37 °C could attain a high ethanol concentration of 82.46 ± 3.42 g/L in the fermentation with the ethanol recovery yield of 81.66 ± 1.88 %; which was 15.37 ± 1.06 % higher than that of the separate hydrolysis and fermentation (SHF) process (70.78 ± 0.25 %). In addition, in the SSF, the process time was shorten to 4 days instead of 7 days in the SHF.

Keywords: bioethanol, cellulose, fractionation, hydrolysis and fermentation, sugarcane bagasse.

1. INTRODUCTION

It was reported that production of sugar released 0.280 ton sugarcane bagasse per every one ton of sugar [1] that contain high portion of cellulose (40 – 45 %) [2, 3]. There are more than 6-7 million tons of sugarcane bagasse annually discharged in Vietnam. Many pretreatment methods were reported that could break down the linkage between three main components of lignocellulose, remove lignin and hemicellulose, decrease the cellulose crystallinity, enhancing the hydrolysis ability of the material cellulose [3 - 6]. However, the remaining lignin in the pretreated materials still affected the hydrolysis and its derivatives inhibited the fermentation in the later step [7]. Another technical question to be solved is to improve the cellulose portion in

the material. Due to the low cellulose content, and the low density of the lignocellulose feedstock, it is difficult to have a high glucose concentration in the hydrolysate then low ethanol concentration in the fermentation [8, 9, 10].

In our previous study, a treatment by organosolv process was developed for sugarcane bagasse to obtain cellulose containing more than 90 % of glucan and only minute amount of lignin [11]. This bagasse can be hydrolyzed at 20 % consistency attained glucose concentration of 154.23 g / L with 77.08 % glucan hydrolysis yield. However, it was observed the inhibition of the enzyme reaction by glucose accumulated in the hydrolysate at a concentration higher than 50 g/L [12] which limit the hydrolysis yield. The materials received from the pretreatment should be easily hydrolyzed by enzymes; the obtained hydrolysate should not contain inhibitors to the fermentation in later step. Besides of that, for economical reason, the process should be done at high biomass consistency (at least 20 %) to balance cost for energy for distillation [8]. With low density of lignocellulose materials, working at high biomass content will be challenging both in mixing in the process and inhibition of the enzyme activity by high glucose released in the hydrolysis [14].

In this study the fermentability of the hydrolysate was verified. The process at high bagasse content that combined hydrolysis and fermentation steps for fractionated bagasse was also investigated in the study aiming to minimize the inhibition of glucose to the hydrolysis, increase the hydrolysis yield and process efficiency.

2. MATERIALS AND METHODS

2.1. Materials

Formic-fractionated sugarcane bagasse: Sugarcane bagasse from the sucrose factory Larusco (Thanh Hoa province) was fractionated with 80 % formic acid at 121 °C for 60 min as previously described [11]. The fractionated bagasse contained 90.00 % glucan and 6.11 % lignin per dry mass. The prepared bagasse with 68 - 70 % wet was stored at -20 °C for further experiments.

Enzyme: NS 22192 (270 FPU/mL) provided by Novozymes (Denmark) was used to hydrolyze the study bagasse at 30 FPU/g dry mass of bagasse.

Saccharomyces cerevisiae Ethanol Red® provided by Fermentis (France) was used for fermentation of the hydrolysate to ethanol following the guide of the supplier.

2.2. Analysis

2.2.1. Glucose determination

Glucose concentration of the hydrolysate was determined using D-Glucose Assay Kit GOD-POD Format (Megazyme) following to the supplier's protocol. Measure the absorbance of the reaction mixtures at 510 nm (Synergy HT Multi-Mode Microplate Reader, Biotek, USA).

Glucose concentration of the hydrolysate was calculated as

$$C_{\text{glucose}} \text{ (g/L)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{standard}} - A_{\text{blank}}} \times f$$

with C_{glucose} : Glucose concentration of the hydrolysate (g/L); A_{sample} , A_{standard} , and A_{blank} : Absorbance of the sample, standard and blank reaction, respectively; f: Dilution factor

2.2.2. Ethanol determination by distillation method

One hundred milliliters of fermentation broth was centrifuged, adjusted to 150 mL by distilled water. Distillation of the solution for 30 min. to obtain 100 ml of distillate. Determine the ethanol content of the distillate by measuring the density at 20 °C according to TCVN 8008:2009.

Ethanol concentration of the fermentation (g/L) was calculated as follows:

$$\text{Ethanol concentration (g/L)} = \frac{\text{Measured ethanol concentration (\%V)} * 78.9/100}{\text{Theoretical ethanol concentration(g/L)}}$$

2.2.3. Determination of ethanol concentration by HPLC

Ethanol concentration was analyzed by Agilent 1200 system equipped with column Aminex HPX-87P according to the provider's instruction. Twenty microliters (20 µl) of fermentation broth was centrifuged at 10,000 rpm for 10 min., filtered through 0.2 µl membrane and injected into the column with mobile phase of 10 mM H₂SO₄ at 60 °C, sample feeding rate 0.5 ml/min. Analysis time was of 45 min/sample. All reagents used for HPLC analysis were of analytical grade.

2.2.4. Yeast biomass density

Biomass density of the culture was estimated by measurement of absorbance at 600 nm (Synergy HT Multi-Mode Microplate Reader, Biotek, USA).

2.3. Experiment design

2.3.1. Separate hydrolysis and fermentation (SHF)

Hydrolysis: Hydrolysis reactions were carried out with 15 or 20 % fractionated bagasse, added with NS 22192 at enzyme dosage of 30 FPU/g dry mass and 0.05 % chloramphenicol to inhibit bacteria. Adjust pH of the mixture to 4.8 using 0.1M citrate buffer. Bring the reaction mixture to 100 mL using 0.1 M citrate buffer. The process was carried out at 50 °C in mixing tubes for 96h. Centrifuge the reaction mixture at 7,000 rpm for 5 min. to receive the hydrolysate.

Fermentation: The hydrolysate contained 121.36 ± 1.3 g/L glucose was added with 1g/L of yeast extract, 4 g/L of urea, 3 g/L of K₂HPO₄ and 2.5 g/L of MgSO₄, autoclaved at 110 °C for 30 min. *S. cerevisiae* (Ethanol Red®) was activated following company's guide and used with 2 g/L for the fermentation. It was fermented for 72h at 30°C. Paralely, the fermentation with 120 g/L glucose added with similar nutrition were carried out to served as control.

Hydrolysis yield was estimated as previously described by Ngo *et al.* (2016) [12]. Fermentation yield was calculated as the percentage of the ethanol formed in the fermentation and theoretical ethanol from glucan of the bagasse in the reaction:

$$\text{Fermentation yield(\%)} = \frac{\text{Ethanol concentration in fermentation broth(g/L)}}{C_{\text{glucose}}(\text{g/L}) * 0.51} * 100\%$$

with C_{glucose} was glucose content in the hydrolysate (g/L), 0.51 was theoretical coefficient of ethanol:glucose conversion.

2.3.2. Pre-hydrolysis and simultaneous hydrolysis and fermentation

Reaction mixture consisted of 20% bagasse in 100 mL mixing tube, supplemented nutrition as above said, autoclaved for 110 °C for 30 min. Let the medium cooled down and added NS22192 at 30 FPU/g dry mass and 0.05 % chloramphenicol to inhibit bacteria. Bring the reaction mixture to 100 mL using 0.1M citrate buffer pH 4.8. Pre-hydrolysis process was done at 50 °C for first 6 h or 24 h. Activated *S. cerevisiae* Ethanol Red® was added with 2 g/L to start the SSF. It was carried out at temperature of 30 °C or 37 °C for 96 h.

Ethanol recovery yield was calculated as:

$$\text{Ethanol recovery yield(\%)} = \frac{\text{Ethanol concentration in fermentation broth(g/L)} * 100}{m * 0.9 * 1.1 * 0.51 * 1000} 100\%$$

of which m was the dry bagasse weight (g) in the reaction, 0.9 was cellulose content of the pretreated bagasse, 1.1 was coefficient converting cellulose to glucose, 0.51 was theoretical coefficient of ethanol: glucose conversion.

3. RESULTS AND DISCUSSION

3.1. Fermentability of the enzymatic hydrolysate

It was reported that bagasse pretreated by either alkaline or acid contained lignin residue, its derivatives or other substances formed during the treatment which later inhibited the ethanol fermentation by the yeast [15]. As the study bagasse contained only an minute amount of lignin, to verify were there the inhibitors to the fermentation presented in the study fractionated bagasse, an enzymatic hydrolysate from 15 % dry mass bagasse (SGB) was used for fermentation by 2 g/L *S. cerevisiae* (Ethanol Red®). The fermentation yield of the hydrolysate was compared to that of a glucose solution at the same condition served as the control (Glc).

It was observed the similar performances in both hydrolysate and glucose fermentations (Fig. 1). The glucose in both reactions decreased rapidly to 0.7 g/L (in the SGB) or not detectable value (in the Glc) after 48 h of fermentation. The fermentation yield in the control attained the maximal value of $93.78 \pm 1.2\%$ at 48 h while that of the SGB reached the maximal value of $91.08 \pm 2.02\%$ at 72 h.

Similarly, the biomass in the Glc sample was maximal at 48 h as in the SGB. The performance of the SGB fermentation showed the hydrolysate of the fractionated bagasse could be easily fermented, gave high fermentation yield of $91.08 \pm 2.02\%$ theoretical yield. Our result seemed higher than reported by both Zha et al. (2012) and Zhao (2012). In Zha report, the highest fermentation yields of 86 % can be obtained after 60 hours of fermentation of hydrolysate of bagasse pretreated by four techniques (2 % H_2SO_4 , 3 % $\text{Ca}(\text{OH})_2$, alkaline/paracetic acid and 72 % H_2SO_4) [16].

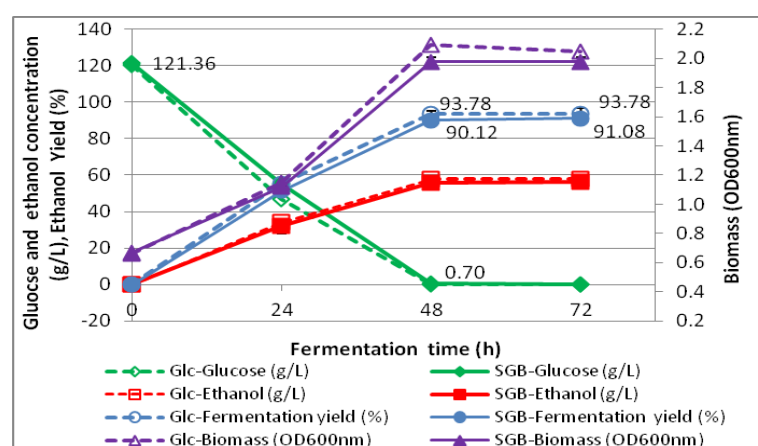


Figure 1. Performance of the ethanol fermentation using sugarcane bagasse hydrolysate (SGB) and glucose media (Glc).

The fermentations were performed in 100 ml bagasse hydrolysate or glucose supplemented with 1 g/L yeast extract, 4 g/L Urea, 3 g/L K_2HPO_4 , 2.5 g/L $MgSO_4$, inoculated with 2 g/L Ethanol Red®, shaking at 120 rpm at 30 °C. Ethanol was analyzed by HPLC as described in Materials and methods.

The detoxification was needed to improve the fermentation yield; treatment of the hydrolysate with $Ca(OH)_2$ at pH 10 for 1h helped improving the fermentation yield to 92 % while that of the undetoxified hydrolysate achieved only 62% [15]. Zhao (2012) obtained 130 g/L glucose in batch hydrolysis with glucan hydrolysis yield of 70 %, ethanol content of 80 g/L and fermentability 82.7 % for 144 h with 20 % formiline fractional bagasse [9]. Comparing to the other treatments, the fermentation of studied bagasse hydrolysate showed there was not inhibitor formed by treatment and the fractionation with formic acid seemed to be the treatment chosen for sugarcane bagasse as alternative glucose resource.

3.2. Simultaneous hydrolysis and fermentation of the fractionated bagasse (SSF)

In the previous study, it was observed the inhibition to the cellulase at glucose concentration higher than 50 g/L. In addition, a high consistency of bagasse in the hydrolysis reaction caused difficulty to the mixing, resulting in inappropriate contact between enzyme and substrate and as the consequence, the hydrolysis yield of the bagasse was lower than that at lower consistencies [12]. Therefore, to achieve high glucose content in the hydrolysate, there will not be a simple increase the bagasse content in the hydrolysis but to an efficient process, it needs to design a suitable hydrolysis technique to achieve both high glucose concentrations in the hydrolysate and high glucan conversion rate within process time.

An interesting process SSF may solve the problems. The process thus can achieve high ethanol content and high ethanol recovery [7, 17, 18]. One of drawbacks of the SSF was in this process; the pretreated biomass was hydrolyzed at the same temperature with the ethanol fermentation. The SSF temperature was recommended not at higher than 37 °C to ensure activity of the yeast and avoid the ethanol evaporation, which was often not the favorable temperature for the cellulase activity, consequently, the hydrolysis process was lower. In addition, biomass was used as unique carbon source in the reaction, thus to facilitate the yeast activity, a pre-hydrolysis was needed to partially liquefy and supply glucose to the yeast at the beginning of the process [4].

In this experiment, SSF reactions with 20 % fractionated bagasse were carried out in mixing tubes at 90 rpm. The biomass were pre-hydrolyzed by NS22192, Novozyme at enzyme dose of 30 FPU/g bagasse at 50 °C for first 6h or 24h before adding 2 g/L Ethanol Red®. The SSF reactions were performed at 30°C or 37 °C for 96h. A separate hydrolysis and fermentation reaction (SHF) with 20 % bagasse was carried out and served as the control. The glucose evolution in the pre-hydrolysis 6h-SSF at 30 °C, Fig. 2A) showed there was 84.03 g/L glucose released in the reaction, equivalent to 42.43 % biomass was hydrolyzed after 6h prehydrolysis. The glucose concentration in the SSF was accumulated to 93.53 g/L at 24 h, and decreased sharply afterwards showing yeast activity converting glucose to ethanol. That also may suggest the SSF can be started earlier to avoid accumulation of the glucose higher than 50 g/L in the reaction [12, 17, 18]. In the SHF process, the glucose concentration in the reaction dropped to zero after 48h of fermentation (Fig. 1). In contrary, in the SSF, at 4 8h, 72 and 96 h, glucose concentration were determined of 2.32 %, 1.03 % and 1.07 % respectively (Fig. 2A), showing the hydrolysis was not yet completed. In SSF process, the biomass of the culture was not increased after 48 h (data not showed), confirmed that the glucose was rapidly convert to ethanol by the yeast. In the process, the hydrolysis occurred parallel with fermentation, the glucose was kept at low concentration, helped preventing reaction from the inhibition of cellulase activity.

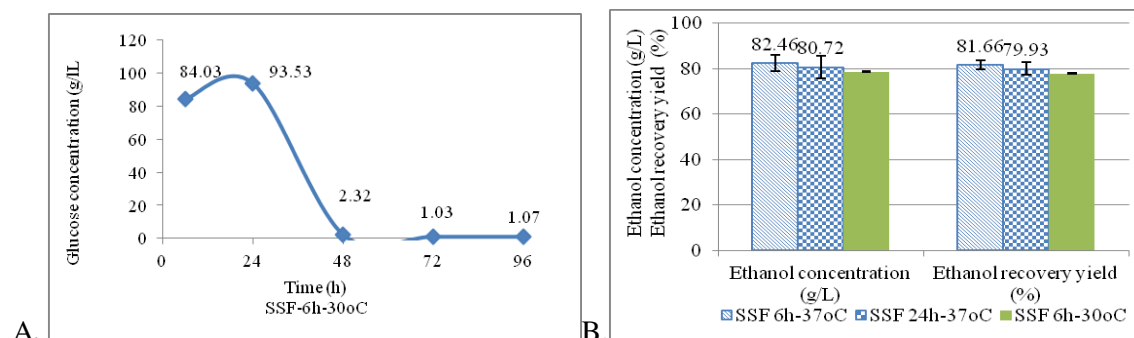


Figure 2. SSF with pre-hydrolysis for first 6h and 24 h and carried out at different temperatures.

- A. Evolution of glucose concentration in the SSF reaction (pre-hydrolyzed for 6h at 50°C, SSF at 30°C);
 B. Ethanol recovery in SSF. Reaction was performed with 20 % dry mass in mixing tubes contained 100ml reaction mixture with enzyme dose of 30 FPU/g. Biomass was pre-hydrolyzed for first 6 h and 24 h at 50 °C. SSF started by adding 2 g/L *S. cerevisiae* Ethanol Red® and carried out at 30 °C and 37 °C for 96 h Ethanol was analyzed by distillation method as described in Materials and methods.

It was seen that for 96h process, the SSF-6h-37°C having ethanol concentration and ethanol recovery higher than those of SSF-6h-30°C (Figure 2.B). In investigation of the influence of temperature on the SSF of cassava starch, Mingjun *et al.* (2012) recognized the balancing between hydrolysis temperature and fermentation played important role in the SSF. Among temperature tested (30 – 40 °C), the highest ethanol recovery was obtained at SSF at 37 °C [19]. At lower temperature, the conversion rate of cellulose to glucose was lower, then the hydrolysis of bagasse may not be completed within process time. This assumption may be the reason for the lowering ethanol recovery of the SSF process at 30°C. SSF-24h-37°C attained parameters lower than that of SSF-6h-37°C (Figure 2B). That may be explained by the higher glucose released in the pre-hydrolysis for 24 h (compared to 6h pre-hydrolysis), causing a severe inhibition to the cellulase. The lower ethanol concentration and ethanol recovery yield obtained in SSF24-37°C in

compared with SSF 6h-37°C also supported to the suggestion of shorten duration of pre-hydrolysis to avoid inhibition to the cellulase reaction.

3.3. Comparison of SSF and SHF processes

The SHF of 20 % bagasse obtained 77.08 % hydrolysis yield (154.23 g/L glucose) [12], fermentation yield attained 91 %, ethanol concentration of 71.47 g/L, ethanol recovery of 70.78 % (Fig. 3). It can be seen clearly in the Fig. 3, comparing two processes SSF-6h-37°C and SHF with 20 % bagasse consistency, the SSF achieved both ethanol concentration and ethanol recovery 15.37 ± 1.06 % higher in compare to SHF process even the SHF process was performed at optimal temperatures for enzyme and the yeast to work according to Novozyme and Fermentis Company guidance. SSF thus gave a very promising solution comparing to SHF: increase ethanol recovery and shorten the process time with a performance very simple.

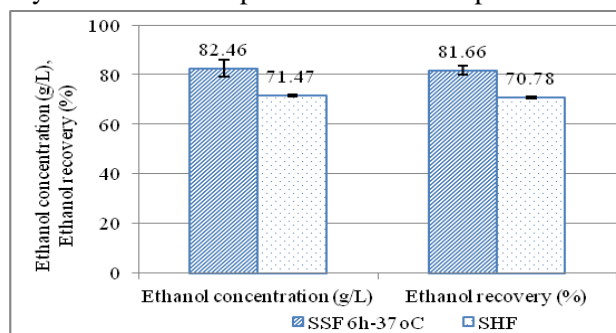


Figure 3. Comparison of 20 % fractionated bagasse SHF and SSF.

Reaction was performed with 20 % dry mass in 100 ml and enzyme dose of 30 FPU/g . Biomass in SSF reaction was pre-hydrolyzed in first 6h at 50 °C then SSF was carried out at 37 °C for 96 h. The hydrolysis step of SHF was done at 50 °C for 96 h and fermentation at 30 °C for 72 h.

4. CONCLUSION

The hydrolysate of fractionated bagasse by formic acid did not contain inhibitors to the ethanol fermentation. The simultaneous hydrolysis and fermentation process can be applied for fractionated bagasse at high consistency, ensuring high ethanol concentration and ethanol recovery within shorter time. Further investigation in fed-batch SSF is now carried out aiming increasing solid consistency.

Acknowledgment. This research was supported by Ministry of Industry and Trading with the Grant No. 05HD-DT.05.13/NLSH under the Research Program of Biofuel.

REFERENCES

1. Chandel A. K., Silva S. S., Carvahol W., Singh O. V. - Sugarcane bagasse and leaves: foreseeable biomass of biofuel and bio-products, J. Chem. Technol Biotechnol **87** (2012) 11–20.
2. Monteiro P. S. A., Selegim P. - Enzymatic hydrolysis of sugarcane bagasse in rotating drum reactor, Florianopolis/SC, 2014.

3. Maryana R., Marifatun D., Wheni A. I., Satriyoa K. W., Angga Rizal W. - Alkaline Pretreatment on Sugarcane Bagasse for Bioethanol Production, *Energy Procedia* **47** (2014) 250-254.
4. Olofsson K., Bertilsson M., Liden G. - A short review on SSF – an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol Biofuels* **1** (2008) 1-14.
5. 5. Yoshida M., Liu Y., Uchida S., Kawarada K., Ukagami Y., Ichinose H., Kaneko S., Fukuda K. - Effects of Cellulose Crystallinity, Hemicellulose, and Lignin on the Enzymatic Hydrolysis of *Miscanthus sinensis* to Monosaccharides, *Biosci Biotechnol Biochem.* **72** (2008) 805–810.
6. Chaturvedi V., Verma P. - An Overview of Key Pretreatment Processes Employed for Bioconversion of Lignocellulosic Biomass into Biofuels and Value Added Products, *Biotech.* **3** (2013) 415–431.
7. Zhao X., Dong L., Chen L., Liu D. - Batch and multi-step fed-batch enzymatic saccharification of Formiline-pretreated sugarcane bagasse at high solid loadings for high sugar and ethanol titers, *Bioresour Technol.* **135** (2013) 350–356.
8. Humbird D., Mohagheghi A., Dowe N., Schell D.J. - Economic Impact of Total Solids Loading on Enzymatic Hydrolysis of Dilute Acid Pretreated Corn Stover, *Economic Impact of Total Solids Loading on Enzymatic Hydrolysis of Dilute Acid Pretreated Corn Stover*, *Biotechnol Prog.* **26** (5) (2010) 1245-1251.
9. Zhao X., Liu D. - Fractionating pretreatment of sugarcane bagasse by aqueous formic acid with direct recycle of spent liquor to increase cellulose digestibility the Formiline process. *Bioresour. Technol.* **117** (2012) 25–32.
10. Chandel A. K., da Silva S. S., Singh O. V. - Detoxification of Lignocellulosic Hydrolysates for Improved Bioethanol Production, *Biofuel Production-Recent Developments and Prospects*, Dr. Marco Aurelio Dos Santos Bernardes (Ed.), InTech, DOI: 10.5772/16454. (2011).
11. Ngo D. S., Pham T. A., To K. A. - Influence of alkaline washing on hydrolysis of formic-fractionated bagasse cellulose, *Journal of Biotechnology* **13** (2) (2015) 345-353.
12. Ngo D. S., Pham T. A., To K. A. - Hydrolysis ability of the formic acid-fractionated bagasse and attempt to increase the glucose concentration in the hydrolysate for feasible cellulose ethanol production, *J. Science and Technology* **111** (2016) 29-36.
13. To K. A., Do T. T., Nguyen X. S., Tran D. M., Le Q. H., Truong P. Q., Do H. T., Nguyen V. Q., Le K. D., Le T., Nguyen M. T. P., Phung T. T. - Saccharification and detoxication of sugar cane bagasse lignocellulose for bioethanol fermentation by an in- house enzyme mixture, *J. Science and Technology* **49** (1A) (2011) 439-445.
14. Humbird D., David R., Tao L., Kinchin C., Hsu D., Aden A. - Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol. Technical Report, NREL/TP-5100-47764, (2011).
15. Jonsson E. J., Alriksson B., Nilvebrant N. O. - Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol Biofuels* **6** (2013) 2-10.
16. Zha Y., Muilwijk B., Coulter L., Punt P. J. - Inhibitory Compounds in Lignocellulosic Biomass Hydrolysates during Hydrolysate Fermentation Processes, *J. Bioprocess Biotech.* **2** (1) (2012) 2-11.

17. Gupta R., Kumar S., Gomes J., Kuhad R. - Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol, *Biotechnol Biofuels* **5** (16) (2012) 1-10.
18. Ferreira V., Faber M.O., Mesquita S.S., Pererra N. - Simultaneous saccharification and fermentation process of different cellulosic substrates using a recombinant *Saccharomyces cerevisiae* harbouring the β -glucosidase gene, *Electron J. Biotechn.* **13** (2) (2010) 1-7.
19. Zhu M., Li P., Gong Y., Wang J. - A Comparison of the Production of Ethanol between Simultaneous Saccharification and Fermentation and Separate Hydrolysis and Fermentation Using Unpretreated Cassava Pulp and Enzyme Cocktail, *Biosci Biotechnol Biochem.* **76** (4) (2012) 671–678.